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Abstract: **OBJECTIVES** This in vitro study aimed to analyze the anti-inflammatory and wound healing potential of green tea extract (GTE) in human gingival epithelial keratinocytes (HGEK) treated with lipopolysaccharides (LPS). **MATERIALS AND METHODS** A cell viability assay was conducted using MTT to determine nontoxic levels of GTE on immortalized HGEK. Cells were concomitantly treated with LPS (1 µg/ml) and GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml, and 10 mg/ml) to assess inflammation. Gene expression levels of inflammatory markers IL-β1, IL-6, and TNFα were measured by RT-PCR and their protein production was assessed by ELISA. The scratch wound healing assay was used to investigate the effects of different concentrations of GTE on cell migration. We also explored the effect of GTE on the induction of the Nrf2/HO-1 pathway in the cells with or without LPS. **RESULTS** GTE at concentrations of 2.5 mg/ml, 5 mg/ml, and 10 mg/ml significantly enhanced cell viability ($p < 0.05$). And IL-β1, IL-6, and TNFα gene expression presented up to 10-fold decrease compared with LPS-treated cells, which was also similarly found on the protein levels. At the same concentrations, cell migration increased. **CONCLUSIONS** The mechanism results showed that GTE produced the anti-inflammatory response by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and increasing the level of antioxidant protein heme oxygenase-1 (HO-1). **CLINICAL RELEVANCE** GTE may be potentially used as oral rinse anti-inflammatory drug for treatment and prevention of oral inflammatory diseases, which is shown here by the ability to reduce the inflammation and increase in cell migration in a dose-dependent manner.

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Dose-dependent green tea effect on decrease of inflammation in human oral gingival epithelial keratinocytes: in vitro study

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Abstract

Objectives: This in vitro study aimed to analyze the anti-inflammatory and wound healing potential of green tea extract (GTE) in human gingival epithelial keratinocytes (HGEK) treated with lipopolysaccharides (LPS).

Material and Methods: A cell viability assay was conducted using MTT to determine nontoxic levels of GTE on immortalized HGEK. Cells were concomitantly treated with LPS (1 µg/ml) and GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) to assess inflammation. Gene expression levels of inflammatory markers *IL-β1*, *IL-6* and *TNFα* were measured by RT-PCR and their protein production was assessed by ELISA. The scratch wound healing assay was used to investigate the effects of different concentrations of GTE on cell migration. We also explored the effect of GTE on the induction of Nrf2/HO-1 pathway in the cells with or without LPS.

Results: GTE at concentration of 2.5 mg/ml, 5 mg/ml and 10 mg/ml significantly enhanced cell viability ($p < 0.05$). And *IL- β 1*, *IL-6* and *TNF α* gene expression presented up to 10-fold decrease compared to LPS-treated cells, which was also similarly found on the protein levels. Same concentrations increased cell migration.

Conclusions: Mechanism results showed that GTE produced the anti-inflammatory response by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and increasing the level of anti-oxidant protein heme oxygenase-1 (HO-1).

Clinical relevance: GTE may be potentially used as oral rinse anti-inflammatory drug for treatment and prevention of oral inflammatory diseases, which is shown here by the ability to reduce the inflammation and increase in cell migration in a dose-dependent manner.

Keywords

Oral rinse, inflammation, green tea extract, lipopolysaccharide, Nrf2, HO-1

Introduction

Growing interest in natural remedies has brought about an increase in scientific reports on substances such as GTE (*Camellia sinensis*). Green tea is a worldwide popular drink that has many compounds containing large amounts of important antimutagenic and anticarcinogenic polyphenols nutrients used medicinally in Asia for 5000 years [1]. Green tea consumption has been associated with health promotion as it is a natural product without side effects [2]. Components of GTE are basically known as polyphenols, such as epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) [3-5]. Among the green tea polyphenols components, EGCG was found to be the most abundant constituent with over 30% of the total catechins [6] and it was shown to reduced dentin surface loss under erosive/abrasive conditions in oral mouth rinsing [7-9]. EGCG has also been suggested to influence the cell anti-inflammatory activity by suppressing the synthesis and inhibiting action of many pro-inflammatory mediators, nitric oxide synthase, peroxynitrite, reactive oxygen/nitrogen species and cyclooxygenase-2 [10-16]. Oral tissue inflammation is primarily initiated by epithelial gingival cells and macrophages when exposed to pro-inflammatory cytokines, interferon- γ or oral bacterial LPS [17]. Activation of macrophage may stimulate the

release of inflammatory mediators also known as pro-inflammatory cytokines (interleukin- β 1, interleukin-6, tumor necrosis factor α) [17, 18]. The production of inflammatory mediators and cytokines in prolonged oral tissue inflammation (i.e. periodontitis) can cause extensive cellular and tissue destruction.

To better prevent or avoid prolonged side effect of oral tissue inflammation, development of more anti-inflammatory agents are still needed as to be employed in treatment of oral diseases. In recent years, the inflammatory-inhibitory potential of naturally derived GTE substances has gained increasing attention. In comparison to steroidal or non-steroidal chemical drugs for treating inflammation, naturally derived substances for preventing prolonged inflammation have limited side effects, fewer intolerance issues and could be available at lower costs than synthetic drugs [10]. Previous studies have shown green tea polyphenols, such as EGCG, to inhibit matrix metalloproteinases -2 and -9; key proteases involved in destructive periodontal diseases [19]. However, the exact role of these EGCG in mediating inflammation on oral tissues is still unknown, since most of previous research has been focusing on the use of green tea EGCC on reducing oral hard tissue erosion-abrasion [7, 9]. As previous studies have focused on the use of green tea extract polyphenols on inflammation in other cell line types and in animal models, it seems expedient to study the application of green tea phenols for their potential to block cytokine-involved inflammatory cascades in oral cells. Further, more knowledge is needed on how these EGCG polyphenols would improve oral tissue wound healing. In addition, other pathways were pointed to assist on progression of inflammation and one of these pathways is the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [20]. The activation of Nrf2 triggered by medication has increasingly gained attention on its possible use in therapies for inflammatory disorders [21, 22] and proved that Nrf2 pathway indeed may play a critical role in inflammation. However, there has been only few Nrf2 activators found to be employed to support oral therapies. Thus, the development of new activating clinical drugs of Nrf2 pathway should be an important goal in the pharmaceutical industry.

Accordingly, the purpose of the present study was to analyze the distinct anti-inflammatory activity and wound healing potential, given the ease of application, of green tea extract topically on the oral epithelium via oral mouth rinse hygiene products. To this end, we have demonstrated the anti-inflammatory potential of green tea extract in human gingival epithelial keratinocytes treated with periodontopathogenic bacterial endotoxin (LPS) by assessing the positive effects on cell viability, wound healing and downregulation of important inflammatory markers. Also, our

findings suggest that GTE could be a strong potential Nrf2 activator for the treatment and prevention of oral inflammatory diseases.

Materials and Methods

Green tea extract solutions

The stock solution of the green tea extract (OM24®, 100% *Camellia sinensis* leaf extract) contained a mixture of catechin concentrations of EGCG (epigallocatechin-3-gallate, 70%), EGC (epigallocatechin, 4%), ECG (epicatechin gallate, 0.6%) and EC (epicatechin, 3%) (Omnimedica, Zürich, Switzerland). The green tea working solution of 1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml was prepared prior to use by mixing GTE powder with 1X phosphate buffered saline (PBS, Sigma-Aldrich). PBS 1X was used as negative control in the cell treatment experiments. This concentration was chosen based on previous studies of dentin erosion using oral mouthrinses containing GTE [7, 9, 19].

Cell culture and LPS-induction

Immortalized human gingival epithelial keratinocytes (HGEK-16) were donated by the Oral Microbiology Institute, Center of Dental Medicine, University of Zurich. The cell line was previously established by transducing E6/E7 oncoproteins from human papillomavirus type 16 to primary cells [23]. Gingival epithelial keratinocytes cells were cultured in an incubator (5% CO₂, 95% humidity at 37°C) and passaged at regular intervals depending on their growth characteristics using 0.25 % trypsin (Seromond Biochrom, Berlin, Germany) and maintained in complete epithelial medium consisting of defined keratinocyte serum free medium (Gibco, 10744-019), supplemented with 100 U/ml penicillin (Sigma-Aldrich, 15140-122), 100 mg/ml streptomycin (Sigma-Aldrich, 15140-122), 2 mM L-glutamine (Sigma-Aldrich, G7513), and 0.25 mg/ml fungizone (Sigma-Aldrich, 15290-018). Medium was changed every 3 to 4 days and cells passaged once a week. The cells used in this study were between the fifth and fifteenth passage. There were six treatments used for this study where the gingival epithelial keratinocytes were induced inflammation: 1) negative control (PBX 1X), 2) positive control (1 µg/ml LPS from *P. gingivalis*, Sigma-Aldrich); 3) HGEK treated with 1 µg/ml LPS and 1 mg/ml GTE, 4) HGEK treated with 1 µg/ml LPS and 2.5 mg/ml GTE, 5) HGEK treated with 1 µg/ml LPS and 5 mg/ml

GTE and 6) HGEK treated with 1 µg/ml LPS and 10 mg/ml. GTE The cells were incubated for 24 h and cell-free supernatant was used for the *IL-β1*, *IL-6* and *TNFα* gene expression assay.

Cell viability assay

HEGK cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma–Aldrich) dye reduction assay (5 mg/ml in PBS 1X). Gingival epithelial keratinocytes (0.1×10^6 cells/ml) were re-cultured in 12-well plates supplemented with fresh medium containing different concentrations of GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml). After an exposure time of 24 h, the solutions were aspirated and the cells washed with PBS 1X before culture medium was newly added. At 24 h after exposure to the respective GTE solution, 500 µl of MTT was added to each well and incubated for 4 h at 37 °C in the dark. In the next step, MTT was removed by aspiration from the wells and isopropanol was added (200 µl, 1N HCl) to solubilize the MTT-formazan crystals formed. The test absorbance at 570 nm and reference absorbance at 630 nm was measured using a spectrophotometer plate reader (Corning Costar, Corning, NY, USA). Experiments were performed in triplicate.

Real-time quantitative polymerase chain reaction analysis (RT-PCR)

Total ribonucleic acid was isolated using Trizol reagent and RNeasy Mini kit (QIAGEN), 24 h after GTE exposure in different concentrations (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and LPS (1 µg/ml). Primer sequences for genes encoding *IL-β1*, *IL-6*, and *TNFα* were designed from Primer3 (version 0.4.0) as follows: *IL-β1* (forward primer: 5'-TAGAGCTGCTGGCCTTGTTA-3', reverse primer: 5'-ACCTGTAAAGGCTTCTCGGA-3'), *TNFα* (forward primer: 5'-TGCCTATGTCTCAGCCTCTT-3', reverse primer: 5'-GAGGCCATTTGGGAAGTTCT-3'), *IL-6* (forward primer: 5'-ATGAACTCCTTCTCCACAAGC-3', reverse primer: 5'-GTTTTCTGCCAGTGC CTCTTTG-3') and GAPDH (forward primer: 5'-GCTCTCTGCTCCTCCCTGTT -3', reverse primer: 5'-CACACCGACCTTCACCATCT -3'). Following Trizol extraction, RT-PCR analysis was performed using 15 µl final reaction volume of TaqMan's One step Master Mix kit (Applied Biosystems). Total ribonucleic acid (40 ng) was used per sample well. Each sample contained pooled messenger ribonucleic acid from Trizol extractions collected from the cell cultures exposed with and without LPS + GTE at different concentrations at 24 h. All samples were tested in

triplicates and 3 independent experiments were performed. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression levels relative to GAPDH and normalized to negative control cells.

Enzyme-linked immunosorbent assay (ELISA) – protein expression

Protein levels of inflammatory markers were determined from cell culture supernatant using human IL-1 β (RAB0273), IL-6 (RAB0306) and TNF α (RAB0476) ELISA Kits (Sigma-Aldrich, St Louis, MO, USA) following manufacture's protocol. Briefly, gingival epithelial keratinocytes (0.1×10^6 cells/ml) were re-cultured in 12-well plates supplemented with fresh medium containing different concentrations of GTE in different concentrations (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and LPS (1 μ g/ml) and incubated overnight at 37°C. After incubation time, the cell culture supernatant was collected in preparation for the ELISA.

Scratch wound healing migration assay

To determine the effect of GTE concentrations on wound healing; a scratch-wounded monolayer model was used. The cells were seeded at a density of 0.1×10^6 cells/ml and cultured into each well of a 12-well plate and incubated for 24 h at 37° C until confluent. Prior to the scratch assay, the cells were exposed to 10 μ g/ml of mitomycin C (Sigma-Aldrich) in serum free media for 2 h, which inhibited mitosis of the cells. The wound was produced by scratching with a 10 μ l pipette tip (700-900 μ m in diameter). Following PBS 1X washes to remove cell debris, the remaining adherent cells were divided in 6 treatment groups: 1) negative control (PBX 1X), 2) positive control (1 μ g/ml LPS from *P. gingivalis*, Sigma-Aldrich); 3) HGEK treated with 1 μ g/ml LPS and 1 mg/ml GTE, 4) HGEK treated with 1 μ g/ml LPS and 2.5 mg/ml GTE, 5) HGEK treated with 1 μ g/ml LPS and 5 mg/ml GTE and 6) HGEK treated with 1 μ g/ml LPS and 10 mg/ml GTE. Digital images were captured using a camera-equipped, inverted microscope (Carl Zeiss, Inc., Thorwood, NY, USA) and wound width measurements were subtracted from wound width at time zero to obtain the net wound closure. The distance between edges of injured monolayer were measured by the Image J software (National Institutes of Health, USA) in pixels and wound closure was expressed as the different the width at 0 h and 24 h after wound simulation.

Western blot analysis

Western blotting was performed to determine the protein expression of Nrf2 and HO-1 proteins after cell treatment with LPS and GTE. The cells (0.1×10^6 cells/ml) were seeded in 12-well plate and incubated for 24 h at 37° C. After incubation, the cells were treated with various concentrations of GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and exposed to LPS (1 µg/ml) for 24 h. Protein was extracted with radio-immunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and centrifuged at $12,000 \times g$ for 15 minutes at 4 °C. The supernatant protein was quantified by bicinchoninic acid assay (BCA, Thermo Fisher Scientific, Rockford, USA) and stored at -80 °C. Total lysates were resolved in SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane and incubated with primary antibodies and the corresponding secondary antibodies. Immune complexes were visualized by the use of an enhanced chemiluminescence western blotting system (BioRad, Richmond, CA). Antibodies used for immunoblotting were as follows: antibody against Nrf2 (ab137550), HO-1 (ab90492) and GAPDH (ab9484) (Abcam).

Statistical analysis

The mean values and standard deviations were computed for the cell viability test and multiple comparisons were conducted by analysis of variance (ANOVA) followed when appropriate by Bonferroni post hoc tests with a global significance level of 5 % to assess the statistical significance of the differences between the experimental groups (SPSS version 22.0, Munich, Germany). All the in vitro experiments were performed in triplicate and from three independent experiments unless otherwise mentioned. Differences were considered significant at $p < 0.05$.

Results

Effect of GTE on HGEK cells viability

The cell viability, as a preliminary study, was shown to be significantly enhanced after GTE exposure at concentrations 2.5 mg/ml, 5 mg/ml and 10 mg/ml compared with the cells cultured without treatment ($p < 0.05$ at 24 h) and with LPS treatment by performing the cell viability assay. Based on these results and previous studies, the green tea extract concentration of 1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml were selected for further experiments. GTE at

concentrations 2.5 mg/ml, 5 mg/ml and 10 mg/ml showed significant increase of maximum 1.5-fold compared to untreated control, while 1 mg/ml did not increase viability (Figure 1).

Green tea extract down regulation of markers for inflammation

To investigate whether GTE could inhibit LPS-induced inflammatory gene expression of *IL-β1*, *IL-6* and *TNFα* cells were simultaneously pretreated with GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and LPS (1 μg/ml) for 24 h. The results showed that LPS (1 μg/ml) treatment remarkably increased by almost 10-fold of *IL-β1*, *IL-6* and *TNFα* compared with untreated cells (Figure 2A). Thus, LPS treated keratinocytes showed the highest level of all pro-inflammatory cytokines and modulators tested in this study. Further, the positive control LPS (1 μg/ml) was used as a standard to obtain GTE inflammatory inhibition activity toward *IL-β1*, *IL-6* and *TNFα* expression. GTE at concentrations 2.5 mg/ml, 5 mg/ml and 10 mg/ml decreased all pro-inflammatory cytokines tested in this study compared to each positive control 8-fold change decrease) compared to the positive control (LPS 1 μg/ml) (Figure 2A). The protein levels of IL-β1, IL-6 and TNFα by ELISA analysis were also shown significantly decreased on GTE test cell groups (GTE at 2.5 mg/ml, 5 mg/ml and 10 mg/ml and LPS at 1 μg/ml) compared to the positive control (LPS 1 μg/ml) ($p < 0.05$) confirming the RT-PCR results (Figure 2B).

Scratch wound healing assay (cell migration)

HEGK cells exposed to GTE at concentrations of 2.5 mg/ml, 5 mg/ml and 10 mg/ml similarly elicited increase in cell migration at 24 h compared to untreated negative control and positive control (LPS 1 μg/ml) (up to 40 %). Wound closure was observed almost complete after 24 h in the presence of GTE at 2.5 mg/ml, 5 mg/ml and 10 mg/ml. Positive and negative controls showed around 80% incomplete healing patterns at 24 h (Figure 3).

Mechanism analysis via Nrf2 signaling pathway

In order to understand whether GTE could exert its anti-inflammatory effect through activating Nrf2 pathway, the downstream protein HO-1 of Nrf2 pathway were investigated in this study. Compared with LPS-stimulated HEGK cells, GTE at 10 mg/ml increased Nrf2 and HO-1 gene expression and protein production (* $p < 0.05$, Figure 4A and B).

Discussion

The purpose of this study was to ascertain the anti-inflammatory and wound healing stimulating effect of green tea extract in human gingival epithelial keratinocytes cells challenged with LPS. Our findings sustain the hypothesis that GTE is a powerful anti-inflammatory agent and in a dose-dependent condition would elicit wound healing cell migration; with the majority of these properties attributed to the green tea plant's polyphenolic compounds, i.e., catechins in the leaves. Consistent with our results, other studies reported biological response in other cell types, such as, antioxidant, antimicrobial, and anti-inflammation effects of EGCG, one of the green tea's compounds [24-26, 10-14]. Green tea also has been shown antimicrobial activity against most of oral bacteria. Additionally, it may enhance oral peroxidase activity and prevent the establishment and progression of periodontitis [27-29]. Based on its anti-inflammatory and antioxidant effects, green tea EGCG could be considered for treatment of innumerable diseases, including neurological diseases, diabetes and hypertension [30-32]. GTE compounds can suppress nuclear factor 'kappa-light-chain-enhancer' of activated B-cells and downstream signaling inflammatory mediators (nitric oxide, inducible nitric oxide synthase, cyclooxygenase-2, prostaglandine-E2) *in vitro* and could be responsible for its significant anti-cancer or chemopreventive activity [33]. Our results (Figure 1) suggested that GTE at concentrations 2.5 mg/ml, 5 mg/ml and 10 mg/ml have a positive effect on oral gingival keratinocytes cell viability by almost duplicating viability compared to the non-treated control. However, it was shown that green tea EGCG could provoke increased cytotoxicity in cells when applied at higher concentrations. An EGCG concentration higher than 100 μ M was reported to cause production increase of H_2O_2 and oxidative DNA damage, whilst concentrations more than 200 μ M EGCG could even affect cell-cycle progression [34, 35]. Consequently, the concentration of EGCG in the test green tea solution should be carefully controlled for in vitro assay applications. In this study, green tea solutions containing maximum of ≈ 50 μ M EGCG concentration were observed to be safe based on the cell viability test (Figure 1). GTE solutions at 2.5 mg/ml, 5 mg/ml and 10 mg/ml concentrations downregulated the expression of inflammation markers *IL- β 1*, *IL-6* and *TNF α* at 24 hours (Figure 2A) and reduced protein production of the same analyzed genes (Figure 2B). *IL- β 1*, *IL-6* and *TNF α* were decreased in gene expression compare to control and the combination with GTE (at 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and LPS (1 μ g/ml) still showed the positive effects of GTE by reducing inflammatory status. LPS is an important component of the outer membrane of Gram-negative bacteria, which

promotes cellular signal transduction through toll-like receptors and secretion of pro-inflammatory interleukins, eicosanoids and nitric oxide [36]. As seen in cells treated only with LPS (Figure 2A and B, darker columns), the gingival keratinocytes treated with lipopolysaccharide here resulted in a significant increase of *IL- β 1*, *IL-6* and *TNF α* gene expression and protein production compared to the negative control. This is a predicted biological reaction to LPS bacterial endotoxin, specially from the highly oral pathogenic bacteria *P. gingivalis*. A decrease in inflammation was also observed in previous in vitro studies using different cell lines, which showed GTE's potential for use as anti-inflammatory drugs by providing its ability to reduce the production of nitric oxide, cyclooxygenase-2, interleukin-6, interleukin-1 β , and tumor necrosis factor α in active macrophages [37-39]. Tumor necrosis factor α is responsible for increased in levels of inflammation by up-regulating other pro-inflammatory cytokines (e.g., interleukin-6, interleukin-1) and consequently inducing angiogenesis and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells transcription, and stimulating nitric oxide production [40]. Tumor necrosis factor α has been targeted by anti-inflammatory screening agents due to its multiple roles in inflammation [41]. Interleukin- β 1 induces secretion of interleukin-6 and interleukin-8, which also plays a role as pro-inflammatory cytokines and is important for the initiation and increase of the inflammatory response to microbial infection [42]. In addition, GTE anti-inflammatory effect was confirmed in animal studies on LPS-induced retinal inflammation [43, 44].

Regarding the possible wound healing effect of green tea via our in vitro scratch wound healing assay, increase difference to the control was found with the GTE at concentrations 2,5 mg/ml, 5 mg/ml and 10 mg/ml at 24h, whilst 1 mg/ml stimulation was non-significant. Despite GTE component's EGCG association with wound healing in pharmacological animal studies [45, 46], in vitro studies with cancer cells showed that EGCG significantly decreased or inhibited cell migration in a dose-dependent manner [47, 48]. Based on our experimental observations, normal gingival keratinocytes showed an \approx 50% increase in cell migration or changes in migrating behavior after exposure to GTE (Figure 3). In contrast to its effect on normal keratinocytes, GTE component's EGCG was found to be specially antiproliferative and apoptotic on cancer cells [48, 49-52]. However, it has been suggested that these effects of EGCG are cancer-specific and EGCG shows pronounced growth inhibitory effect on cancerous cells, but not on normal cells [53]. Another study demonstrated that in cultured skin keratinocytes, EGCG acts to enhance differentiation without triggering apoptosis [54]. Topical application of EGCG has been identified and reported earlier on human skin results on increased cell proliferation and reduced keratinocyte apoptosis [55], however to the best of our knowledge there is no report of green tea on oral cells.

Our results suggest that oral gingival epithelial keratinocytes presented significant decrease in cell mobility/migration activity challenged with *P. gingivalis* LPS (1 µg/ml) at 24 h (Figure 3). Moreover, GTE at concentrations 2,5 mg/ml, 5 gm/ml and 10 mg/ml could successfully stimulate cells in an inflammatory environment when exposed to LPS. The unchanged migration found after 1 mg/ml GTE exposure could be explained by its lower concentration and EGCG content, which reduces green tea stimulation potency. And it indicates that there is possibly a threshold for gingival keratinocytes responsiveness to green tea. We also have to consider that any difference in green tea effects on migration likely reflects relative sensitivity of specific cell types to LPS. But further examinations of molecular basis are still needed to support this hypothesis. Regarding the signaling pathway analysis, our results showed that GTE may activate Nrf2 pathway bringing into play its anti-inflammatory effect on cells exposed to LPS (Figure 4A). Also, the Nrf2-dependent anti-oxidant gene HO-1 was also increased (Figure 4B). Some reports previously confirmed that the activation of Nrf2 pathway prevents LPS-induced upregulation of pro-inflammatory cytokines, such as IL-β1, IL-6 and TNFα analyzed here [20, 56]. Drugs used to activate Nrf2 pathway could be considered for use on potential oral therapies for oral inflammatory diseases, such as gingivitis or periodontitis. Until today there are few Nrf2 activating medicaments used in the dental clinic. Therefore, GTE could be used to develop new and safer Nrf2 activator for clinical use in dentistry.

This study has taken a step in the direction of defining the effect of green tea extract on inflammation suppression associated with wounding. However, the results neither be viewed, nor are they presented as conclusive. In addition, it is important to emphasize that problems in methodological research design of in vitro assays place limitations on interpretations. Further, the potential for green tea catechins to aid in epithelial formation bears further study. Although this study did not support the finding of earlier animal studies [57], other research designs may yet show a benefit in its use. In addition, in vivo angiogenesis and granulation tissue augmentation by GTE have been demonstrated [30]. To our knowledge, there is a lack on studies evaluating GTE's effect on oral epithelial healing. Therefore, further animal model research is needed.

Conclusions

In summary, our results sustained the hypothesis that GTE attenuates the inflammation in gingival epithelial keratinocyte treated with LPS by downregulating inflammatory cytokines in a

dose-dependent manner. The results from our experiment support that GTE can be considered a potent anti-inflammatory agent with a potential on oral therapeutic against inflammation.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical Approval

This article does not contain any studies with human participants performed by any of the authors.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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Figure Captions:

Fig. 1. Increase in cellular activity of keratinocytes cells 24 h after exposure. A significant increase in keratinocyte cellular activity was detected for GTE at 2.5 mg/ml, 5 mg/ml and 10 mg/ml compared to non-treated cells (control). The o in the figure indicates the outliers. Data is shown of 3 samples (3 wells each) * $p < 0.001$.

Fig. 2. GTE stimulated downregulation of inflammatory markers and protein production of same markers on gingival keratinocytes exposed to LPS. (A) RT-PCR analysis shows gene expression decrease of *IL-1 β* , *IL-6* and *TNF α* under simultaneous exposure to GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and LPS (1 μ g/ml) compared to control (white bars). (B) ELISA results showed decrease in translational production of inflammatory proteins *IL-1 β* , *IL-6* and *TNF α* in the cells treated with GTE and LPS compared to control (white bars). * $p < 0.05$. Mean \pm standard deviation.

Fig. 3. Induction of keratinocytes cell migration on in vitro scratch wound healing assay after GTE (1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml) and LPS (1 μ g/ml) exposure. Representative images are shown from 3 independent experiments and light gray areas define the areas lacking cells (Scale bar 300 μ m). Images were analyzed using ImageJ software to calculate wound area. Data is expressed as the mean values of percentage wound closure relative to the corresponding 0 h time point and represent the mean percentage closure \pm standard deviation ($n = 3$): * $p < 0.05$ vs. time-matched treated control for 12 h and 24 h.

Fig. 4. The effect of GTE on the activation of Nrf2 and the expressions of HO-1 in LPS stimulated oral keratinocytes. The total proteins of the cells were prepared and the expressions of Nrf2 (A) and HO-1 (B) were analyzed by Western blot. Results are expressed as mean \pm standard deviation of three independent experiments ($N = 3$). * $p < 0.05$.